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LONG TERM PRESERVATION OF DNA FROM HONEY BEES (*APIS MELLIFERA*) COLLECTED IN AERIAL PITFALL TRAPS

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Abstract.—This study examines the preservation of nuclear and mitochondrial DNA from honey bee (*Apis mellifera* L.) specimens which were first kept in propylene glycol-based antifreeze under various conditions, and then stored long-term, refrigerated in 95% ethanol. Two sets of bees were subjected to the propylene glycol treatment, then ethanol storage. One set consisted of bees captured in the field in propylene glycol-containing "aerial pitfall traps", where they remained for up to 21 days. A second set consisted of bees taken from a hive and kept in propylene glycol under various temperature and lighting conditions for up to 90 days. Both the field bees and laboratory bees were then stored long-term in ethanol before evaluation of the persistence of nuclear and mitochondrial DNA using the polymerase chain reaction. DNA integrity was preserved for both field and laboratory specimens. The results demonstrate that propylene glycol-captured, ethanol-preserved honey bees retain both nuclear and mitochondrial DNA after capture and long term preservation. It is suggested that with little or no modification, the techniques described here might be applied to other studies involving trap-collected arthropod specimens.

Newly developed molecular genetic analysis techniques can provide unique insights into the complex interrelationships among organisms, but they are dependent on collection and preservation techniques that adequately preserve DNA in the collected specimens. Numerous studies on field-collected organisms have been conducted using polymerase chain reaction (PCR) based techniques to describe both individuals and populations (reviewed by Caterino et al. 2000). With the increased availability of techniques to genetically characterize living organisms using PCR and other molecular genetic tools, the question has arisen of how to best preserve field-captured specimens in order to keep their DNA intact. This is important in ecological studies of arthropods in which field collection techniques are passive and may require long, unattended sampling periods. In such studies, immediate transfer of the insects or other specimens to a suitable long-term preservative is often

impractical. Studies involving new DNA preservation methodologies have shown promise, e.g., studies of Coleoptera (Reiss et al. 1995), and Odonata (Logan 1999). However, many of those methods are not compatible with several common field-sampling techniques.

One common field technique of passively capturing arthropods and other invertebrates is the utilization of the pitfall trap. The pitfall trap employs a liquid preservative medium to capture specimens which fall into a partially buried trap that has been placed in the appropriate habitat. Pitfall traps are efficient and cost effective for passively capturing insects for many ecological studies, and have been used extensively for population studies of numerous ground-dwelling arthropods, including arachnids, formicids, Coleoptera and most notably carabids (Southwood 1978; Post et al. 1993). Rubink et al. (1990) reported the use of aerial-style, pitfall traps, herein referred to as "aerial pitfall traps" for the capture of foraging Hymenoptera, particularly honey bees (*A. mellifera* L.). Pitfall traps may be baited with a suitable attractant (Rubink et al. 1990), and are provisioned typically with a capture medium such as alcohol, ethylene glycol (EG), or a soapy water solution into which the insects fall, and are killed and preserved. Propylene glycol (PG) has recently been used in place of EG because of the former's more "environmentally friendly" nature (Greene 1996).

Recent developments in genetic analysis utilizing microsatellites and mtDNA have brought the use of passive trapping systems into a new light. However, limited information is available on suitable preservation techniques for subsequent molecular genetic analyses of invertebrate species. This is because most studies use preservation media developed for immediate field preservation of specimens, which would not be appropriate for use as a capture medium in a passive trapping system such as the pitfall trap (Post et al. 1993; Reiss et al. 1995; Dillon et al. 1996; Cooper 1998; Dawson et al. 1998). Thus, questions remain about the longer-term viability of DNA from insects collected in pitfall style traps containing a capture medium such as PG. In this study, bees kept in PG under either simulated or actual field conditions are used to assess the preservation over time of both nuclear DNA (microsatellites) and mtDNA (cytochrome b gene). Herein it is suggested that the DNA of arthropods collected in PG-containing pitfall traps can be preserved adequately for characterization by PCR.

MATERIALS AND METHODS

Two separate sets of honey bees were subjected to treatment and DNA analysis. In the first set, the "laboratory bees", bees were collected from a single hive, placed in three Nalgene containers (simulated aerial pitfall traps) containing PG antifreeze (Lowtox®, 92% PG, 5% water, and 3% proprietary additives; Prestone Corp., Danbury, CT) and then subjected continuously to one of the following three conditions: 20°C or 40°C with normal laboratory lighting, or ambient outdoor temperature (11-27°C) and natural sunlight. Bees from each of the above treatments were removed at 5, 20, and 90 days post sampling, transferred to 95% ethanol, and stored for up to four months at 4-6°C before subsequent DNA analysis. At the time of sampling the living bees from the hive, several "control" bees were also placed directly into 95% ethanol and refrigerated. Preliminary observations made during the course of this work, as well as the work of others have shown that cold storage of insects in 95% ethanol was sufficient to preserve DNA (Post et al. 1993).

A second set of bees, the "field bees", was comprised of 10 field-collected specimens obtained from aerial pitfall traps in conjunction with honey bee population studies at Welder Wildlife Refuge, San Patricio Co., Texas. Honey bees were collected at three-week intervals from aerial pitfall traps containing PG antifreeze, placed into 95% ethanol, and stored at 4-6°C until analyzed. The honey bees used were a small random subset of bees captured in various aerial pitfall traps at Welder Wildlife Refuge between August 2000 and February 2001.

After storage in ethanol for up to 4 months at 4-6°C, individual bees were taken from the refrigerator, and their abdomens and wings were removed. The remainder of each bee was put into a 1.5 mL microcentrifuge tube and crushed thoroughly using a large pipet tip which had its pointed end rounded by partial melting. DNA was then extracted from the bee tissues by using a QIAamp® DNA Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions for DNA extraction from tissues. The period of time the tissues were subjected to the lysis treatment during the DNA extraction procedure varied from 2 to 16 hours in different experiments without any obvious effect on the DNA yields. At the end of each DNA extraction procedure, the DNA from a single bee was dissolved in 200 µL of the elution buffer supplied in the QIAamp® DNA Mini Kit.

The nuclear DNA loci amplified using PCR were the highly polymorphic A7 and the less polymorphic A24 microsatellite loci (Estoup et al. 1993; 1995). PCR conditions were based on those of Cornuet and colleagues (Estoup et al. 1995), but with some modifications as indicated below. PCR mixtures contained 25 μ L total volume which consisted of the following components: 1X *Taq* DNA polymerase buffer (Promega Corp. Madison, WI), 1.2 mM $MgCl_2$, 0.2 mM of each deoxyribonucleoside triphosphate, 0.32 μ M of each primer, 1 μ L of undiluted template DNA from the Qiagen protocol mentioned above, and 1.25 unit *Taq* DNA polymerase (Promega Corp.). The primers for amplification of the A7 and A24 loci were described previously (Estoup et al. 1994; 1995). After an initial denaturing stage of 3 min at 94°C, 30 cycles of 94°C for 30 sec, 57-59°C for 30 sec and 72°C for 30 sec were performed. This was followed by a 10 min final extension at 72°C. An annealing temperature of 59°C was used for amplifications of the A7 locus of the laboratory bees (Fig. 1a). For all other PCR amplifications of nuclear DNA, an annealing temperature of 57°C was used. Reactions were done using hot-start PCR by withholding primers from the reaction mixture until the temperature reached 94°C. Using hot start conditions reduced the amount of primer dimers that were obtained, which in turn, increased the yield of the major desired bands. Aliquots of 10 μ L were run on 14% polyacrylamide gels in Tris-Borate EDTA running buffer (Sambrook et al. 1989). Gels were stained with ethidium bromide and visualized under UV light.

A 485 base pair (bp) section of the cytochrome b gene of mtDNA was amplified using primers suggested by Crozier et al. (1991). The PCR amplifications were performed in 25 μ L total volume containing 0.5X *Taq* DNA polymerase buffer (Promega Corp.), 1.5 mM $MgCl_2$, 0.2 mM of each deoxyribonucleoside triphosphate, 0.5 μ M of each primer, 1 μ L of undiluted template DNA from the Qiagen protocol mentioned above, and 1.25 unit *Taq* DNA polymerase (Promega Corp.). The PCR temperature profile was 94°C for 3 min followed by 25 cycles of 94°C for 15 sec, 50°C for 15 sec, 72°C for 5 sec, followed by an additional extension step of 72°C for 10 min. PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide, and visualized under UV light.

RESULTS

Nuclear microsatellite DNA from the A7 and A24 loci, and mtDNA from a 485 bp segment of the cytochrome b gene were all amplifiable, to varying degrees, depending upon the laboratory or field conditions to which they had been exposed.

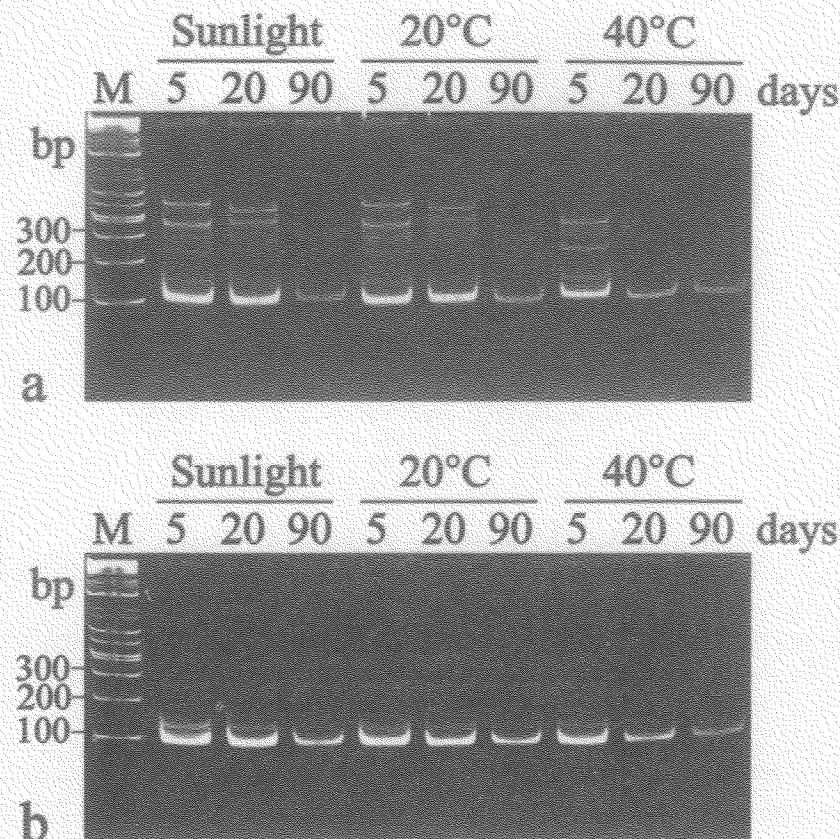


Figure 1. (a) PCR amplification of the A7 microsatellite locus from laboratory bees. DNA was isolated from individual bees collected from PG-containing simulated pitfall traps exposed for various lengths of time to sunlight and ambient temperatures, or to laboratory lighting and constant 20°C or 40°C. This DNA was amplified by PCR and the products run on 14% polyacrylamide gels. Lane M contains a molecular weight standard. (b) PCR amplification of the A24 microsatellite locus. Same experimental conditions as for panel (a).

Figure 1 shows typical results from the PCR amplification of the A7 and A24 loci from the laboratory bees. The data clearly show that all samples contain amplifiable DNA, even from bees kept in PG for up to 90 days at 40°C. The major band in each lane is approximately 100 bp in length, which is within the expected range for both of these polymorphic loci (Estoup et al. 1995). There is generally a decrease in the amount of PCR product with increasing time of treatment of the bees in PG, with the most pronounced drop-off typically seen in the 90-day samples.

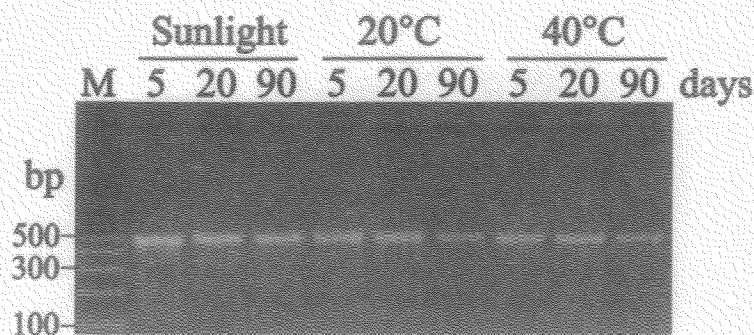


Figure 2. PCR amplification of a 485 bp section of the cytochrome b gene of mtDNA from laboratory bees. Same experimental conditions as for Fig. 1 except that samples were run on a 2% agarose gel.

Preliminary observations made during the course of this study revealed that the outcome of positive and negative controls were as expected. That is, for DNA from positive control bees which were put in ethanol immediately after collection from the hive (i.e., without any incubation period in PG), amplification results obtained were similar to those shown in Figure 1. Additionally, PCR amplifications performed without template (negative control) gave no products corresponding to the 100 bp or larger bands seen in reactions done with template present. Figure 2 illustrates that mtDNA showed similar viability in samples as shown for the nuclear DNA, with amplifiable DNA being retained throughout the 90-day period of PG treatment.

Figures 1 and 2 illustrate the typical results of the PCR amplification of DNA from the laboratory bees. Amplifications using either nuclear DNA or mtDNA as template from PG-stored laboratory bees treated variously for 20 days or less was essentially always successful, yielding amplification products in more than 99% of reactions (127/128 attempts) using DNA prepared from 31 different laboratory bees. For laboratory bees treated in PG for 90 days, PCR amplifications were typically still successful, but there were exceptions (Table 1). As seen in Table 1, for the nuclear DNA analyses, 10 of the 11 bees (91%) kept in PG for 90 days yielded DNA which could be successfully amplified. The table also shows that for the mtDNA, all of the 90-day treated bees yielded amplifiable DNA, although successful amplifications did not occur in every reaction attempt.

Table 1. Amplifiability of DNA from laboratory bees preserved in PG for 90 days.

Treatment	Bee #	Frequency of Successful Amplifications ^a	
		Nuclear DNA	mtDNA
Sunlight	L1s	2/2	2/2
	L2s	2/2	2/2
	L3s	2/2	2/2
20°C	L1t	0/2	1/2
	L2t	1/2	2/2
	L3t	3/3	2/2
	L4t	4/4	3/3
40°C	L1f	1/2	2/2
	L2f	1/2	2/2
	L3f	3/3	1/2
	L4f	4/4	3/3

All amplifications of all laboratory bees subjected to 90 days of treatment in PG are reported here.

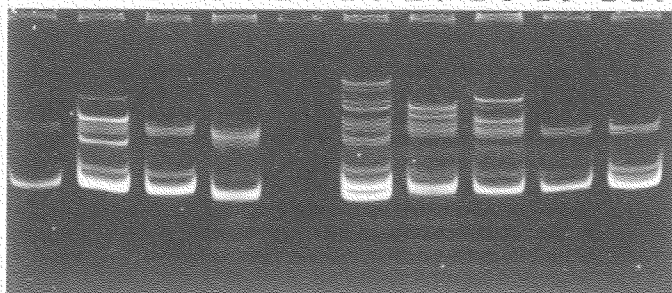
^a These values represent the number of successful amplifications over the number of amplifications attempted.

Figure 3 shows results from the PCR amplification of the A24 locus from 10 different bees collected from aerial pitfall traps in the field. A major band of about 100 bp appears in these PCR amplifications also, as expected. Only one of the DNA preparations made from the 10 different bees failed to give any amplification (bee F5). DNA from the remaining bees gave successful amplifications. Results were again similar for the A7 locus (data not shown). Mitochondrial DNA amplification was successful for the same 9 of 10 bees, and gave similar quantities of amplification products (Fig. 4).

DISCUSSION

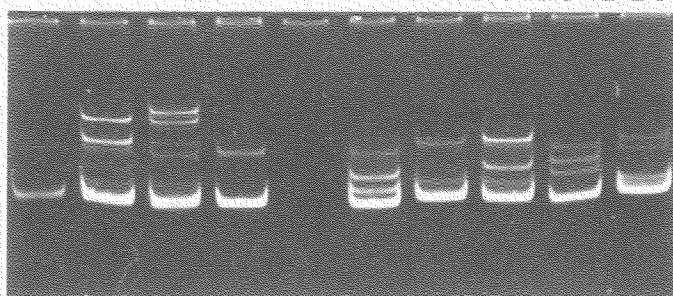
This work provides experimental evidence that propylene glycol will adequately preserve both nuclear DNA and mtDNA in honey bees collected under typical field conditions. The field bees in this study were kept as long as 21 days in PG, and 90% of them yielded amplifiable nuclear and mitochondrial DNA. For laboratory bees kept under simulated pitfall trap conditions up to 20 days, 100% of them (31 bees) yielded amplifiable nuclear and mitochondrial DNA. For laboratory bees kept for 90 days in PG under various conditions, 91% of the bees yielded amplifiable nuclear DNA, and 100% yielded amplifiable mtDNA. Thus, PG appears to be an excellent choice for a preservative for bees collected by passive methods.

bee # F1 F2 F3 F4 F5 F6 F7 F8 F9 F10



a

bee # F1 F2 F3 F4 F5 F6 F7 F8 F9 F10



b

Figure 3. (a) PCR amplification of the A7 microsatellite locus from field bees. DNA was isolated from individual bees from various PG-containing scout traps in the field, from which collections were made at 21-day intervals. This DNA was amplified by PCR and the products run on 14% polyacrylamide gels. (b) PCR amplification of the A24 microsatellite locus. Same experimental conditions as for panel (a).

Dillon et al. (1996) reported that EG preserved DNA in two species of parasitic wasps, and it seems likely that these results would be similar for PG. However, the reduced environmental hazards exhibited by PG make it the medium of choice for further studies of this sort, as well as for general studies using liquid-filled passive trapping systems.

Other studies have used ethanol for long-term preservation of specimens (e.g., Reiss et al. 1995). The current study corroborates the use of 95% ethanol and refrigeration as a highly adequate semi-permanent storage medium. The field bee specimens had been stored in refrigerated ethanol medium for 1-4 months after field collection and

bee # F1 F2 F3 F4 F5 F6 F7 F8 F9 F10

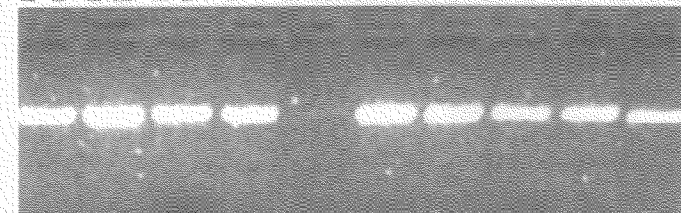


Figure 4. PCR amplification of a 485 bp section of the cytochrome b gene of mtDNA from field bees. Same experimental conditions as for Fig. 3 except that samples were run on a 2% agarose gel.

before DNA extraction and analysis. In cases where it is possible to place specimens directly into ethanol or another long-term storage medium (acetone has also been used, as well as a number of other materials, and cryogenic storage), one of those should be acceptable. However, the techniques tested here show promise for many field-oriented studies where direct transfer of specimens to a long-term storage medium is impossible or impractical. It is expected that with little or no modification, the techniques described here might be applicable to other studies involving trap-collected arthropod specimens.

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OBSERVATIONS ON NEST SITE SELECTION AND LITTER SIZE IN THE GRAY SHREW (*NOTIOSOREX CRAWFORDI*) FROM PRESIDIO COUNTY, TEXAS

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Abstract.—This study reports on nest site selection and litter size in the gray shrew *Notiosorex crawfordi*, from two locations in Presidio County, Texas, Shafter Mine (SM) and Bunton Flat (BF) which lie in the northern region of the Chihuahuan Desert. This is the first study on these parameters for *N. crawfordi* from this region of Texas. At both sites active shrews were most frequently observed under decaying leaves of lechuguilla (*Agave lechuguilla*) and Torrey yucca (*Yucca treculeana*) which had fallen to the ground, as well as under mesquite (*Prosopis juliflora*) and creosote (*Larrea divaricata*) shrubs. All nests were found under decaying leaves of lechuguilla or Torrey yucca. Nests were constructed out of grass stems and leaf fragments and had a small, central cavity ranging from 1.8 - 3.0 cm in diameter. Most nests (68.6%) contained two side-openings, while the remainder had only one. Litter sizes ranged from 3 - 5 at SM ($n = 7$) as compared to 1 - 3 at BF ($n = 12$).

The gray shrew (*Notiosorex crawfordi* Coues) occurs in the southwestern United States, ranging from southern California and eastward to west Texas (Davis 1941; Borell & Bryant 1942; Punzo 2003a; Whitaker 1996). It is typically found in arid and semiarid habitats including desert scrub, canyon washes, arroyos and creek beds (Dixon 1924; Coulomb & Banta 1964; Duncan & Corman 1991). Although they may be locally abundant, they are rarely encountered in the field. Consequently, little is known concerning the behavior, ecology, or natural history of this shrew (Armstrong & Jones 1972; Simons et al. 1990; Punzo 2003a). Previous field studies on this secretive animal have included observations on its nesting habits (Gander 1928; Hoffmeister 1986) as well as its relative abundance and preferred habitat at several sites in Oklahoma (Baker & Spencer 1965), southeastern Arizona (Simons et al. 1990; Duncan & Corman 1991; Simons & Van Pelt 1999), west Texas and northern Mexico (Baker 1962; Judd 1969), and southern California (Cunningham 1956). What little data are available show that *N. crawfordi* typically constructs nests under decaying vegetation (Simons et al. 1990), under stacks of lumber and other debris in areas of human habitation (Hoffmesiter & Goodpaster